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14. ABSTRACT

Prostate cancers that recur after so-called androgen ablation therapy ('CR-CaP') are typically more aggressive, more likely to spread to local lymph nodes and bones, and less likely to respond to second-tier treatments, and therefore, contribute to significantly decreased patient survival. We posit that enzymes called Src-family kinases (SFK) are required for the progression to CR-CaP, and thus, targeting these enzymes should prevent CR-CaP formation to suppress their growth. We will use animal models of human and mouse CR-CaP in conjunction with genetic and biochemical experiments to show that SFK are critical to the formation of CR-CaP, and thus, are therapeutically targetable using SFK-specific drugs. Our important pre-clinical studies on the critical role played by SFK in CR-CaP disease will serve as the foundation to establish immediate clinical trials in which CaP patients are treated with drugs such as KX2-391 at the commencement of androgen-deprivation therapy.

15. SUBJECT TERMS
Src, androgen receptor, castration recurrent, human xenografts, src and akt isoforms

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Introduction:

From Initiating PI:

We are studying the role of Src-family kinases (SFK) in promoting castration-recurrent prostate cancer (CR-CaP) using genetic and pharmacological approaches along with several animal models of CR-CaP. Our synergistic collaboration is based the expertise of the initiating PI (Gelman) in the molecular signaling of SFK in cancer progression, combined with the expertise of the partnering PIs in the CWR22 and TRAMP CR-CaP mouse models (Mohler and Smith, respectively), and in the role of neuroendocrine cells (NE) in the progression of CR-CaP (Smith).

Body:

The role of my laboratory in the *Synergistic Research Program* was to provide expertise in NE-differentiation in animal models of prostate cancer, and to provide models for *in vivo* therapeutic evaluation of inhibitors *of src* family kinases *(SFKs)* in primary xenografts of human prostate cancer as a preclinical model. Our experimental studies were performed in primary cultures of prostate endothelial cells and primary xenografts of human prostate cancer tissue established from fresh surgical specimens of radical prostatectomy tissue. Importantly, this pre-clinical model is unique for analysis of the effects of SFK inhibitors on the individual prostate cellular compartments located within a human tissue microenvironment, including a human microvasculature. We characterized the presence of the individual SFK isoforms in primary cultures of human prostate endothelial cells during Year 1 and in Year the focus was on evaluation of the role of specific SFK isoforms in human prostate endothelial cells in vitro and the effects on prostate endothelial cells and the adjacent cancer epithelium in the intact prostate ti ssue microenvironment of the primary xenografts of human prostate cancer tissue.

The endothelial cells in the prostate of rodents or in tumors of human prostate cancer cells passaged in culture and transplanted to immuno-compromised rodent hosts, are fundamentally different from the endothelial cells in human prostate cancers because the rodent endothelial cells do not express AR. Consequently, the involution and recovery of the endothelial cells in all rodent models reflect secondary effects mediated via the prostate cancer cells, and not the endogenous primary response of the endothelial cells which we have demonstrated to be characteristic of human prostate endothelial cells. Therefore, all experimental efforts in our portion of the *Synergistic Research Program* were focused on validation of human endothelial cell models that allow evaluation of the role of SFKs in the biology of human prostate cancer, and prostate endothelial cells as potential organ specific targets for systemic treatment with SFK inhibitors, in the clinically relevant model of fresh surgical specim ens of human prostate cancer tissue. The human model systems employed were: 1) primary cu ltures of human prostate endothelial cells harvested from fresh surgical specimens; and 2) primary xenografts of fresh prosta te tissue transplanted intact to immuno-compromised hosts implanted with a source of testosterone to provide serum levels of androgen comparable to humans. The goal of this project was to evaluate the differential effect of inhibitors of SFKs on the homeostasis and angiogenic potential of prostate endothelial cells in prostate cancer, and the consequences of androgen-deprivation on the prostate microenvironment. Our working hypothesis was that the role of endothelial cells in the overall response to chemotherapy has been co mpletely overlooked, even though endothelial cells represent a/the key barrier to the uptake of chemotherapeutic agents and circulating androgens, and that the state of the endothelial barrier may dramatically affect the progression to castration-recurrent disease. The corollary to this hypothesis is that this question c ould never by explored in rodent models because of the significant biological difference in prostate endothelial cells between humans and rodents. Our experimental work was focused on the realization that the endothelial cells are the first cells in the prostate to encounter systemically administered SFK-inhibitors, and recent studies that demonstrate that SFKs play a critical role in the trans-cellular transport of serum components through the endothelial cells, thereby affecting both the survival/proliferation of the endothelial cells as well as their transport/barrier functions. Furthermore, there is important clinical data that demonstrates that tumor vasculature often reacts in an opposite manner as to what is expected, or does not react at all, to current anti-angiogenic therapies (including TKIs), the so called "vascular steal phenomenon". Therefore, understanding the response of prostate endothelial cells to SFK-inhibitors may provide valuable insight into their ultimate availability to chemotherapeutic agents of prostate

cancer epithelial cells, both androgen-stimulated and castration-recurrent, as well as predicting the importance of SFKs in endothelial cells as chemotherapy targets relative to solely targeting SFKs in cancer epithelial cells.

1, **Gene expression profile of freshly isolated populations of human prostate endothelial cells harvested from benign prostate tissue and from prostate cancer tissue specimens.**

Fresh surgical specimens of un-involved prostate tissue (benign tissue), and prostate cancer tissue (verified by the Department of Pathology to be >70% cancer), were obtained within two hours of delivery of the excised prostate to Pathology, the tissue enzymatically disaggregated (a 1.0 hr procedure), and the endothelial cells isolated immediately by two rounds of fluorescence activated cell sorting (FACS). Viable endothelial cells were identified by expression of CD31 and exclusion of actinomycin. RNA was isolated, amplified, and gene expression profiled by array-analysis using Illumina gene expression arrays.

This data set has continued to mature throughout the life of the project, and represented the major experimental activity during the extension period without funding. During the final interval of the project additional endothelial cell populations from benign and CaP tissue were characterized, as well as a small number of specimens from benign prostate tissue from African American CaP patients (N = 4)(no cancer tissue was available). Table 1 presents the final summary of gene expression profiling of genes involved in SFK-mediated signaling from endothelial cells isolated from benign prostate tissue and CaP tissue from Caucasian American patients, endothelial cells isolated from benign prostate tissue from African American CaP patients, and intact benign prostate tissue and CaP tissue (all from Caucasian American CaP patients).

TABLE 1. RELATIVE GENE EXPRESSION IN HUMAN PROSTATE ENDOTHELIAL CELLS ISOLATED BY FACS FROM FRESH SURGICAL SPECIMENS OF PROSTATE CANCER TISSUE AND UNINVOLVED PROSTATE TISSUE

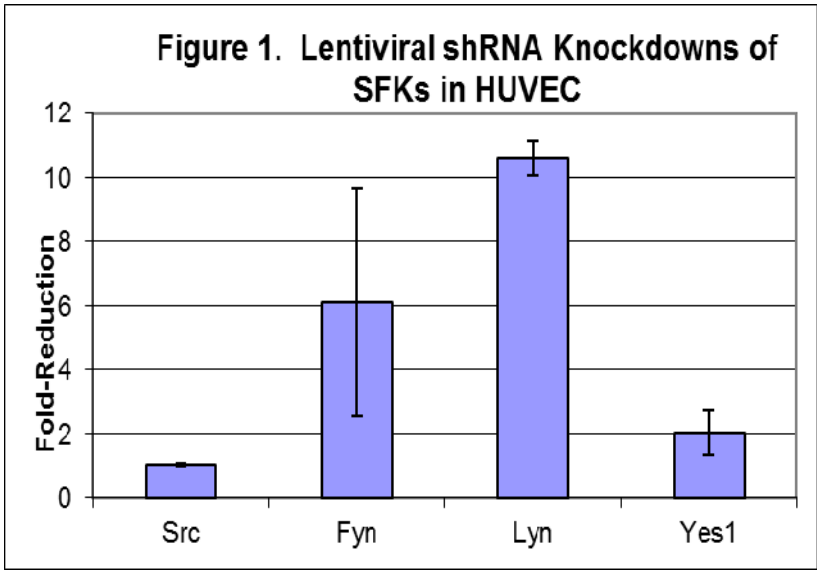
GENE SYMBOL		ECs from Benign Tissue from AA	ECs from Benign Tissue from CA	ECs from Cancer Tissue from CA	CaP Tissue	Benign Prostate Tissue	DEFINITION
SRC		9.0	4.2	4.7	30.6	21.4	v-src sarcoma viral oncogene homolog
FYN		238.1	212.8	204.7	16.9	18.2	FYN oncogene related to SRC, FGR, YES
YES1		293.5	260.9	491.4	715.5	702.1	v-yes-1 Yamaguchi sarcoma viral oncogene
LYN		623.3	528.5	476.1	62.0	44.5	v-yes-1 Yamaguchi sarcoma viral related oncogene
AKAP12		149.3	124.5	330.8	101.0	154.8	A kinase (PRKA) anchor protein 12 (gravin)
AKT1		687.4	884.1	737.1	792.4	668.4	v-akt murine thymoma viral oncogene homolog 1
AKT2		ND	ND	ND	ND	ND	v-akt murine thymoma viral oncogene homolog 2
AKT3		9.4	23.2	11.7	17.8	19.9	v-akt murine thymoma viral oncogene homolog 3
NOS3		4.3	ND	1.5	ND	ND	nitric oxide synthase 3 (endothelial cell)
CD36		270.6	163.9	177.4	ND	2.6	CD36 molecule (thrombospondin receptor)
CD47		550.6	556.3	410.9	963.4	1232.3	CD47 molecule (thrombospondin receptor)
CAV1		722.5	673.7	1105.6	916.8	1406.4	caveolin 1
CAV2		153.2	89.3	187.5	157.6	269.4	caveolin 2
KDR		27.7	ND	3.8	ND	ND	vascular endothelial cell growth factor receptor 2
FLT1		123.7	44.2	87.3	11.6	10.4	vascular endothelial cell growth factor receptor 1
PDGFRA		ND	ND	ND	451.1	555.9	platelet-derived growth factor receptor, alpha
PDGFRB		2.2	ND	ND	96.6	73.0	platelet-derived growth factor receptor, beta
THBS1		1816.7	2054.4	2182.1	342.9	476.3	thrombospondin 1
PIK3CA		30.6	45.8	43.3	50.5	46.7	phosphoinositide-3-kinase, catalytic, alpha
PIK3R1		243.1	269.7	210.7	115.5	171.3	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
HSP90		3093.6	4221.1	4590.9	2478.9	2382.0	heat shock protein 90kDa (cytosolic)
HIF1A		1165.5	1242.6	1240.9	842.2	777.8	hypoxia-inducible factor 1
EPAS1		436.7	327.9	519.1	171.0	258.8	hypoxia-inducible factor 2
PSA		ND	ND	ND	713.7	578.3	androgen receptor
FOLH1		ND	ND	ND	1238.1	463.1	prostate specific membrane antigen

ND = Not Detectable; ECs = Endothelial Cell; AA = African American; CA = Caucasian American

Gene expression profiling of the FACS-isolated endothelial cells, and of total RNA from benign prostate tissue or prostate cancer tissue (>90% cancer epithelial ce lls) was performed using an Illumina array platform. As anticipated, there were marked differences in the relative level of expression of individual genes between the FACS-sorted endothelial cells and the intact prostate tissue comprised predominantly of epithelial cells. The prostate tissue specimens demonstrated strong expr ession of PSA and FOLH1 (PSMA), with no measurable expression in the endothelial cell isolates. Prostate endot helial cells expressed the majority of genes involved in SRC-family signaling at levels comparable to prostate tissue, validating their potential as a target for SFK-inhibitors. However, it is of interest to note there is marked differential expression of several targets for TKIs between prostate endothelial cells and prostate tissue, with endothelial cells expressing higher levels of FYN, LYN, KDR, FLT1, HIF1 and HIF2. In contrast, prostate tissue expressed higher levels of SRC, YES1, PDGFRA and PDGFRB. Consequently, the endothelial and epithelial compartments should demonstrate marked differences in response to targeted TKIs.

2. **shRNAs specific for individual isoforms of SFKs FYN and LYN negatively affect human prostate endothelial cell function.**

The gene expression profiling suggested that SFK members FYN and LYN would represent better therapeutic targets than SRC and YES1 for interdiction of prostate endothelial cell function. Evaluation of the effect of shRNAs specific for individual members of the SFKs gene expression demonstrated clearly that inhibition of FYN or LYN activity was significantly more effective than was inhibiting expression of either SRC or YES1. The lentivirus vectors carrying the shRNAs for the four SFK isoforms were obtained from Dr. Gelman (PI of Synergistic Award) through his capacity as Director of the shRNA Core Resource at RPCI. Figure 1 demonstrates that shRNAs for both Fyn and Lyn knocked down mRNAs levels in HUVEC by 6.2-fold and 10.6-fold, respectively. However, the shRNAs for SRC and YES1 only reduced mRNA levels by 1.0-fold and 2.0-fold, respectively.



Subsequently, the effect on endothelial tube formation (by HUVEC angiogenesis) of shRNAs specific for inhibition of expression of each of the four major isoforms of SFKs was evaluated using the same shRNAs as used for the gene expression knock-down studies. HUVEC was employed as a surrogate for primary cultures of prostate endothelial cells to establish experimental conditions for analysis of tube formation before implementation of the assay using the more demanding primary culture-based system. Figure 2

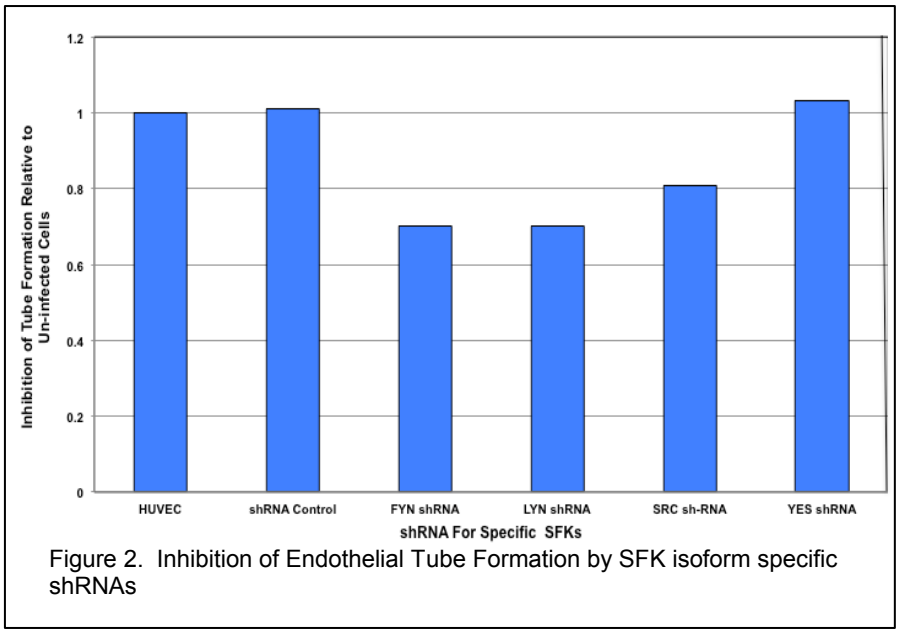
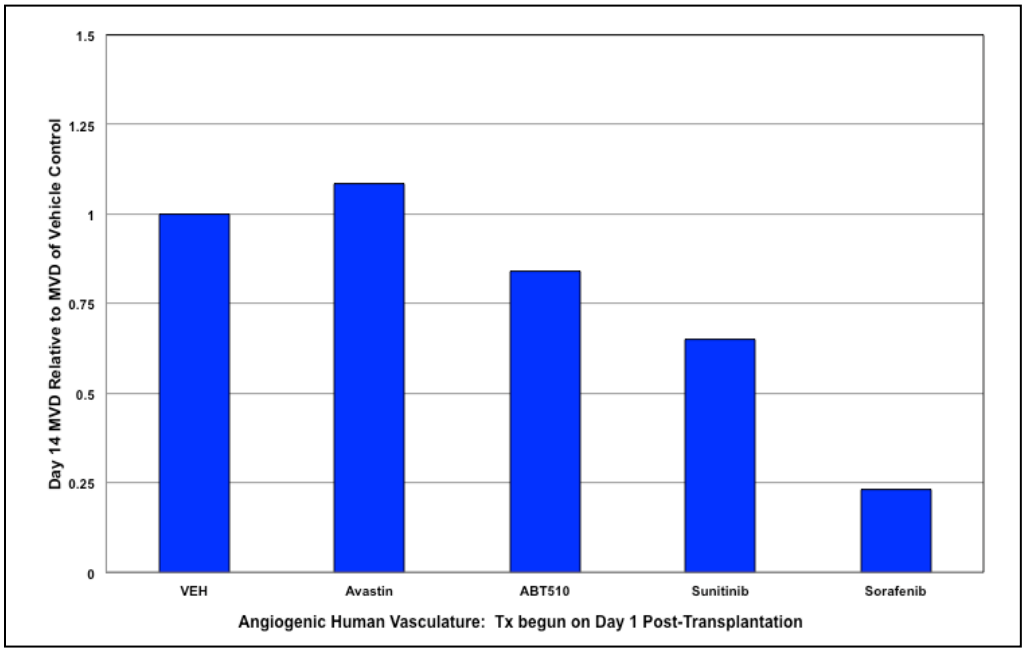


Figure 2. Inhibition of Endothelial Tube Formation by SFK isoform specific shRNAs

demonstrates that the individual isoforms of the SFKs differentially regulated endothelial tube formation by HUVEC (all assays were done in the presence of androgen supplementation). It should be noted that the shRNA to block YES1 expression, the most highly expressed SFK isoform, not only did not inhibit tube formation, but had a small stimulatory effect, suggesting YES1 negatively regulated caveolae-mediated signaling in HUVEC. FYN and LYN were the predominant SFK isoforms expressed in prostate endothelial cells and were the principal targets for shRNAs that perturbed mediation of signaling associated with "angiogenesis" in the HUVEC endothelial tube formation assay.

3. <u>SFK-inhibitors perturb human angiogenesis in primary xenografts of human prostate</u>.

Based upon the *in vitro* studies that demonstrated specific shRNAs that block signaling mediated through specific SFKs (LYN and FYN) could inhibit directly endothelial cell function (angiogenic activity = tube formation in MATRIGEL), the effect of broad spectrum receptor tyrosine kinase (TKI) inhibitors on angiogenesis by human prostate endothelial cells in pr imary xenografts of human prostate tissue was evaluated. Fresh surgical specimens of human prostate tissue were transplanted into immuno-compromised mouse hosts that had been pre-implanted with sustained release testosterone pellets to maintain human serum levels of testosterone. Treatment of the hosts with the TKIs began on the day after transplantation of the human prostate tissue. Microvessel density in the xenografts was evaluated on Day 14 post-transplantation at the conclusion of the period of active angiogenic activity induced by transplantation. Host animals were treated with four different anti-angiogenic agents that are in routine clinical use: a) avastin – a humanized antibody for VEGF; b) ABT-510 – a thrombospondin mimetic peptide; and c) two broad spectrum TKIs, Sunitinib and Sorafinib. The dose and route for the anti-angiogenic agents were: ABT510 - 30mg/kg (i.p.) 2x daily (a gift of Abbott Laboratories); Avastin - 10mg/kg every 3 days (i.p .); Sunitinib - 10mg/kg daily (gavage); Sorafenib - 10mg/kg daily (gavage). Avastin and the thrombospondin-mimetic ABT-510 had marginal effects on angiogenic activity by the endogenous human vessels in the primary xenografts. In contrast, both of the TKIs, presumably through direct effects on SFK members FYN and/or LYN, the SFK isoforms expressed differentially in human prostate endothelial cells, signi ficantly inhibited human angiogenesis in the primary xenograft model. The inhibitory activity was not due to actions mediated through the VEGF-R since avastin did not inhibit angiogenesis as a monotherapy. Because of the differential expression of FYN/LYN versus SRC/YES1 in prostate endothelial cells relative to the pattern of expression in prostate epithelial cells, this suggests the potential for therapeutic approaches that target differentially/independently the two compartments: the prostate tissue microenvironment versus the prostate epithelial cell/CaP cell compartment.



Key Research Accomplishments

-Characterization of the gene expression profiles of human prostate endothelial cells from prostate cancer tissue and from matched benign prostate tissue. The unique aspect of this characterization is that the gene expression profiling was done on cells freshly isolated from surgical specimens and purified by fluorescence activated cell sorting. Consequently, the gene expression profile reflects the in situ situation and does not reflect adaptation to culture.

-Demonstration that human prostate endothelial cells express a different pattern of SFKs than do prostate epithelial cells, and that shRNAs for only those SFKs that are expressed by the human prostate endothelial cells modulate the biological activity of human prostate endothelial cells.

-Demonstration that TKIs inhibit angiogenesis by human prostate endothelial cells in vivo, and that the inhibition is not modulated through inhibition of VEGF-R because Avastin does not induce a comparable level of inhibition.

-Demonstration that therapeutics that target specifically FYN or LYN could have significant clinical utility for management of human prostate cancer through i nhibition of angiogenic activity by the prostate endothelial cells.

Reportable Outcomes
None

Conclusion
The striking conclusion suggested by the experimental observations of this study is that because the endothelial cell compartment and the cancer epithelial ce ll compartment of human prostate cancer express different profiles of SFKs, therefore, the two compartments should respond differently to therapeutic modalities that target specifically individual isoforms of the SFK. This exquisite differential sensitivity to targeted TKIs should allow modulation of the endothelial cell mediated vascular barrier, and/or inhibit angiogenesis, allowing enhanced access of novel therapeutic modalities to the prostate cancer epithelial compartment, potentially with minimal collateral systemic effects.

References
A portion of this work was presented as both a poster and a podium presentation at the CDMRP Innovative Minds in Prostate Cancer Research (IMPaCT Conference) in Orlando, Fl in March 2011.

Appendices
None